Investigating the endobacteria which thrive in arbuscular mycorrhizal fungi

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Running head: The Endobacteria Thriving in Arbuscular Mycorrhizal Fungi

Summary

The study of the so-called "unculturable bacteria" is still considered a challenging task. However, the current availability of culture-free approaches and the improvement in their sensitivity allows the identification and characterization of such microbes in complex biological samples.

In this chapter we report how endobacteria thriving inside arbuscular mycorrhizal fungi (AMF), which are themselves obligate biotrophs of plants, can be studied using a combination of *in vitro* culture, molecular biology and microscopy techniques.

Key words: Endobacteria, Arbuscular mycorrhizal fungi, *Candidatus* Glomeribacter gigasporarum, Mollicutes-related endobacteria, Transmission electron microscopy, Fluorescence *in situ* hybridization, Real-time quantitative PCR, Bacterial enrichment

1. Introduction

Thousands of microbes are commonly associated with plant roots, forming the so-called root microbiota which plays a pivotal role on plant life. Among them, a group of soil fungi that colonize the roots of most land plants, the arbuscular mycorrhizal fungi (AMF), has a key role improving mineral nutrition and protection of their host plant. AMF, which belong to the ancient phylum Glomeromycota *(1)*, have been considered as the oldest group of fungi capable to positively interacting with plants: they have been hypothesized to be crucial for the terrestrialization of first land plants c. 450 Mya *(2)*. In addition to some distinctive features (AMF are asexual, multinucleated and obligated biotrophs), Glomeromycota may harbour endobacteria in their cytoplasm *(3)*. Two types of endobacteria have been so far described and identified in AMF: a rod-shaped Gram-negative β-protebacterium called *Candidatus* Glomeribacter gigasporarum (*Ca*Gg) *(4)* and a coccoid bacterium which represents a still enigmatic taxon of Mollicutes-related endobacteria (Mre) *(5)*. Differently from Mre, that show a wide distribution across the Glomeromycota, the presence of *Ca*Gg is limited to the Gigasporaceae family. *Ca*Gg has been deeply investigated: its genome sequence has revealed that the endobacterium is nutritionally dependent on the fungus and has a potential role in providing its host with essential factors like vitamin B12 *(6)*. The fungus, on the contrary, is not obligately dependent on the endobacterium *(7)*. However the removal of *Ca*Gg causes some morphological changes in *Gigaspora* spores and a reduced proliferation of the presymbiotic fungal hyphae. By contrast, information on Mre is extremely limited: based on 16S rRNA gene sequences, this novel bacterial taxon is sister to a clade encompassing the Entomoplasmatales and Mycoplasmatales *(5)* and shows high level of sequence variability. Interestingly, *Ca*Gg and Mre have been simultaneously detected in some *Gigaspora margarita* isolates (fam. Gigasporaceae) hosting what has been described as a new fungal microbiota *(8)*. Thus, single or multiple bacterial populations can thrive inside AMF that, being themselves obligate symbionts, need a host plant to complete their life cycle. Consequently, this complex "Russian dolls-like" organization is difficult to dissect with traditional culture-based techniques. Further, the endobacteria so far described in AMF are considered unculturable microbes, thus they cannot be obtained in pure culture.

In this chapter, we present several techniques that can be applied to investigate the endobacteria thriving in AMF. First, we describe three methods which have been set up to obtain the fungal material and, thereby, their associated endobacteria. The in pot culture is the recommended method for *G. margarita* routine propagation and the obtainment of large amounts of spores. The Millipore sandwich allows to produce clean and intact extraradical mycelium, while the root organ cultures (ROCs) can be used to obtain *in vitro* spores and mycelium. As for bacteria living inside insect *(9)*, the demonstration of the intracellular localization is the first criterion to speak about endobacteria. Therefore, as a second step, we illustrate the protocols to process the biological material for transmission electron and confocal microscopy, in order to detect the endobacteria and describe their morphology. Third, we present some molecular techniques that make possible a direct investigation of the endobacteria from their fungal hosts. Having as a pre-requisite the availability of specific primer pairs, the PCR permits to identify the endobacteria, whereas the real-time quantitative PCR (qPCR) allows to assess their abundance. Fluorescence *in situ* hybridization (FISH) permits to reveal and simultaneously localize the presence of endobacteria in AMF spores. Last, we describe a filtering based protocol first described by Ghignone and colleagues (6) that allows to obtain a spore lysate enriched in the endobacterial component with a limited carryover of fungal nuclei. All the techniques described in this chapter have been applied with success to the study of the endobacteria thriving in AMF *(5-8, 10-12)*.

The presence of endobacteria thriving inside the cytoplasm of fungi has been reported few times so far. In addition to Glomeromycota, endobacteria have been described inside other group of fungi, such as Mucoromycotina. Among them, *Rhizopus microsporus*, a rice pathogenic fungus whose pathogenicity is related to the internal presence of a strain of *Burkholderia rhizoxinica (13)*; *Mortierella elongata*, a filamentous fungus which hosts a Burkholderia-related endobacterium *(14)*; *Endogone*, one of the oldest plant-associated fungi which host Mre in fruiting body-forming spores *(15)*. All these findings strongly suggest that the presence of endobacteria in the cytoplasm of fungi is more widespread than expected. Novel bacterial populations still wait to be discovered and characterized. As a consequence, the application of protocols which allow to unambiguously detect and

identify these bacterial dwellers of fungi will dramatically improve the knowledge of such complex symbiotic systems.

2. Materials

Prepare all solutions using analytical reagents and deionized water (unless indicated otherwise). Use sterile consumables and reagents, or, prior to use, autoclave them at 120°C for 20 min. Wear personal protective equipment, carefully handle dangerous reagents and diligently follow all waste disposal current regulations when disposing of waste materials.

2.1 Biological materials and growing media

The fungal material employed in the experiments (*see* sections **2.1** and **3.1**) consists of monoxenic inocula of the AMF *G. margarita* BEG34, purchased from specialized companies and/or in-house propagated following the in pot culture technique (*see* subsection **3.1.1**).

2.1.1 Pot cultures

1. Plastic pots (0.9 L volume).
2. Sterilized quartz sand (oven-sterilized at 180°C for 3 h).
3. *Trifolium repens* (clover) seeds (*see* **Note 1**).
4. Clean collected AMF spores.
5. Long Ashton fertilization solution *(16)*, modified (low phosphate): 0.75 mM $MgSO_4$ $7H_2O$, 1 mM $NaNO_3$, 1 mM K_2SO_4, 2 mM $CaCl_2$ $2H_2O$, 32 µM Na_2HPO_4, 0.025 mM Fe Na EDTA, 0.005 mM $MnSO_4$ $12H_2O$, 0.00025 mM $CuSO_4$ $5H_2O$, 0.0005 mM $ZnSO_4$ $7H_2O$, 0.025 mM H_3BO_3, 0.0001 mM Na_2MoO_4 $2H_2O$.
6. Sieves (aperture 100 µm).

2.1.2 Spore sterilization

1. Ultrapure water.
2. Chloramine T.
3. Streptomycin sulphate.

2.1.3 *Lotus japonicus* seeds sterilization and germination

1. Sulfuric acid (99.99%).

2. Sterile water.

3. Agar-H_20 (0.6% Agar) plates, diameter 9 cm.

2.1.4 Millipore sandwich method

1. Agar-H_20 (0.6% Agar) plates, diameter 9 cm.

2. Magenta boxes.

3. Sterile cellulose nitrate membranes (pore size 45 µm).

4. Sterilized quartz sand (oven-sterilized at 180°C for 3 h).

5. 1:2 diluted modified Long Ashton solution (*see* subsection **2.1.1**).

6. Sterilized AMF spores.

7. 3-4 days germinating *L. japonicus* seedlings.

2.1.5 *In vitro* fungal propagation under root organ culture (ROC) condition

1. *Cichorium intybus* (chicory) transformed root cultures (*see* **Note 2**).

2. Sterilized AMF spores.

3. Minimal (M) Medium *(17)*: 3 mM $MgSO_4$ $7H_2O$, 0.79 mM KNO_3, 0.87 mM KCl, 1.22 mM $Ca(NO_3)_2$ $4H_2O$, 35.0 µM KH_2PO_4, 21.7 µM Na Fe EDTA, 4.5 µM KI, 30.3 µM $MnCl_2$ $4H_2O$, 9.2 µM $ZnSO_4$ $7H_2O$, 24.0 µM H_3BO_3, 0.5 µM $CuSO_4$ $5H_2O$, 0.01 µM Na_2MoO_4 $2H_2O$, 40 µM glycin, 0.3 µM Thiamin HCl, 0.5 µM Pyridoxin HCl, 4 µM Nicotinic Acid, 277 µM Myo-inositol, 10 g/L Sucrose, Phytagel 4 g/L, pH 5.5.

2.2 Morphological analyses

2.2.1 Transmission electron microscopy

1. Ultramicrotome with glass and diamond knives.

2. 1% toluidine blue (w/v).

3. Polypropylene spot plate (well Ø 2 cm).

4. Hot plate.

5. Uranyl acetate solution: prepare a saturated solution by dissolving uranyl acetate in ddH2O.

6. NaOH (pellets).

7. Lead citrate solution.

- Solution A (Lead nitrate stock solution): add 31.25 g in 500 mL of doubled-distilled water (ddH_2O). Add 10 drops of HNO_3 10N (HNO_3 10N: 630 g in 1 L of ddH_2O).
- Solution B (Sodium citrate stock solution): add 41.50 g in 500 mL of ddH_2O. Add 5 drops of solution A.
- Solution C (NaOH 1N): add 0.2 g in 5 mL of ddH_2O.

Prepare the solution as follows: 2.1 mL solution A + 2.1 mL solution B. Mix. Add 0.8 mL solution C. Mix.

Solution A and B can be stored at room temperature. Solution C must be freshly prepared every time.

8. Philip CM10 transmission electron microscope (FEI, Hillsboro, OR, USA).

2.2.2 Confocal microscopy

1. SYTO 9® Green-Fluorescent Nucleic Acid Stain (Life Technologies, Carlsbad, CA, USA), 5 mM solution in DMSO. Store at -20°C and protect from light. Freshly prepared working solution for bacterial (50 nM–20 µM) and eukaryotic (10 nM–5 µM) cell visualization: make a 1:1000 dilution in ultrapure water. Vortex to mix. Store on ice and protect from light.

2. Leica TCS-SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.3 Molecular analyses

2.3.1 DNA extraction

Rapid DNA extraction

1. 10X PCR buffer (Sigma).

2. Sterile 1.5 mL plastic pestles.

CTAB-based DNA extraction

1. 2X CTAB (Cetyltrimethylammonium bromide) extraction buffer: 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB (w/v). Adjust the volume of the solution with ultrapure water. Autoclave for 20 min at 120°C.

2. Proteinase K: prepare a 1 mg/mL stock solution in ultrapure water. Aliquot and store at -20°C. Prepare working concentration (1 µg/mL) in ultrapure water.

3. RNase A (100 mg/mL). Prepare working solution (40 ug/µL) in ultrapure water following the manufacturer's instructions.

4. Polyvinylpyrrolidone (PVP).

5. Phenol:chloroform:isoamyl alcohol (25:24:1).

6. Chloroform.

7. Cold 2-propanol.

8. 70% ethanol.

2.3.2 PCR

1. PCR reagents: 10 µM of suitable primers (*see* **Tab. 1**), 2.5 mM of each dNTP, 5X Phusion® HF Buffer, Phusion® DNA Polymerase (2U/µL) (Thermo Fisher Scientific, Waltham, MA, USA), ultrapure water.

2. PCR purification and clean-up: Wizard® SV Gel and PCR Clean-Up System (Promega, Fitchburg, Wisconsin, USA).

2.3.4 Cloning

1. Cloning vector: pGEM®-T Easy Vector Systems (Promega, Fitchburg, WI, USA).

2. Chemically competent bacterial cells: One Shot® TOP10 Chemically Competent *Escherichia coli* (Life Technologies, Carlsbad, CA, USA).

3. Fresh LB plates (9 cm diameter) with ampicillin (final concentration 100 mg/mL).

2.3.3 Real-time qPCR

1. 48-well StepOne™ Real time PCR system and StepOne™ software (Life Technologies, Carlsbad, CA, USA) or similar Real time PCR equipment.

2. Qubit® 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA)

3. PCR reagents: 3 µM of each suitable primers (*see* **Tab. 1**), 2X Power SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA), ultrapure water.

2.4 Fluorescence *in situ* hybridization (FISH)

1. Sterile 1X and 10X phosphate buffered saline (PBS), pH 7.2.

2. Fixative solution: 4% paraformaldehyde *(18)*. Heat up 45 mL of ultrapure water at 55-58°C (avoid exceeding 60°C). Add 2 g of paraformaldehyde and stir with a magnet. If necessary, add a few drops of NaOH 10N while stirring continuously until powder dissolves. Add 5 mL of 10X PBS. Cool on ice. Adjust pH to 7.2-7.4 with HCl. Filter the solution with 0.45 µm filter. Store the solution a few days at 4°C, or 2-3 weeks at -20°C. Avoid repeated freeze-thaw cycles.

3. Microscope slides with 8 individual wells (well Ø 6 mm) and large cover slides.

4. Low melting point agarose.

5. 50%, 75% and 100% ethanol.

6. Coplin jars.

7. Hybridization oven.

8. Proteinase K (*see* subsection **2.3.1.2**). Prepare working solution (10 µg/mL) in ultrapure water.

9. Tween 20.

10. Suitable labeled oligonucleotide probes (*see* **Tab. 2**). Probes are 5'-end labeled with fluorochromes like fluorescein isothiocyanate (FITC) or cyanine dies (Cy3 or Cy5). Resuspend in ultrapure water the probes to obtain a 500 ng/µL probe stocks. Aliquot and store the stocks in the dark at -20°C. Dilute probe stocks at the working concentration of 50-70 ng/µL with ultrapure water and aliquot in individual tubes (50 µL per tube) to avoid repeated freeze-thaw cycles. Store in the dark at -20°C.

11. Sterile 20X saline-sodium citrate (SSC) buffer (3 M NaCl and 0.3 M sodium citrate, pH 7). Aliquot and store at -20°C.

12. 100% formamide. Aliquot and store at -20°C (*see* **Note 3**).

13. 50X Denhardt's solution: dissolve the Denhardt's powder in ultrapure water according to the manufacture's instruction. Store at -20°C for up to two years. Prepare 25X concentrate dilutions as working solution. Aliquot and store at -20°C.

14. Antifade mounting medium: 1,4-Diazabicyclo[2.2.2]octane (DABCO) solution (25 mg/mL). Dissolve 250 mg DABCO in 10 mL 1X PBS. Add 90 mL Glycerol. Adjust pH to 8.6 with HCl or NaOH. Store the solution at 4°C.

15. Leica TCS-SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.5 Bacterial enrichment

1. 0.9% NaCl in ultrapure water (w/v). Autoclave for 20 min at 120°C.

2. Sterile 1.5 mL plastic pestles.

3. Sterile 3 µm cellulose nitrate filters.

4. Sterile syringes and syringe filter holders.

5. RQ1 RNase-Free DNase (1 U/µL) (Promega, Fitchburg, WI, USA).

3. Methods

3.1 Biological materials and growing media

3.1.1 Pot cultures

The in pot culture is the recommended method for *G. margarita* routine propagation and for the obtainment of large amounts of spores, since it represents a "nearly natural" method to obtain AMF material under controlled conditions.

1. Place the oven-sterilized quartz sand in 0.9 L pots.

2. Soak the sand with the modified low phosphate Long Ashton solution and let it drain. Inoculate 100 *G. margarita* spores under the sand surface by pipetting.

3. Spread 80-100 *T. repens* seeds on the sand surface and cover them with a thin sand layer.

4. Put the pots in a climatic chamber with a photoperiod of 16 h and a temperature of 23°C during the day and 21°C during the night. Keep the pots in culture for at least 3 months.

5. During the entire culturing period, fertilize the pots once a week with the modified low phosphate Long Ashton solution. Water the pots with water whenever needed.

6. After 3 months of cultivation, collect the newly formed spores from the sand soil by putting a 100 mL aliquot of substrate in a beaker and adding water. Shake the beaker so that spores are temporarily kept in suspension and immediately pour the water in a 100 µm aperture sieve. Recover the sieve content in a large glass Petri dish by washing it with water. Manually collect the spores under a stereomicroscope by pipetting with a P1000 pipette or by individually collecting them with laboratory tweezers (*see* **Note 4**).

3.1.2 Spore sterilization

The entire procedure should be performed under a biological hood.

1. Prepare a solution containing 3% chloramine T and 0.03% Streptomycin sulphate in ultrapure water. Shake well until the powders are completely dissolved. Typically, 50 mL of solution are prepared to sterilize up to 2000 AMF spores.

2. Place the collected spores in a tube and remove the residual water. Add the sterilization solution and shake the tube. Typically, 1.5 mL of sterilization solution is used for 100 spores (*see* **Note 5**).

3. Place the tube horizontally, so that spores are not pelleted at the bottom, and wait 10 minutes.

4. Remove the sterilization solution by pipetting and add the same volume of ultrapure water; shake well and wait 5 minutes.

5. Remove the water by pipetting and add the same volume of sterilization solution. Shake well, place the tube horizontally and incubate 10 minutes.

6. Remove the sterilization solution by pipetting and add the same volume of ultrapure water; shake well and wait 5 minutes.

7. Remove the water by pipetting and add the same volume of ultrapure water; shake well and wait 10 minutes.

8. Repeat step 7. After having waited 10 minutes, remove by pipetting all traces of water.

Spore are now ready for subsequent treatments. If their intended use is DNA extraction, place tubes in liquid nitrogen and store the frozen spores at -80°C until use. If they will be employed for further vital manipulation, store them at 4°C for at most one week.

3.1.3 *Lotus japonicus* seeds sterilization and germination

The procedure should be performed under a chemical hood and wearing suitable gloves until step 4, since sulfuric acid is toxic and corrosive.

1. Extract *L. japonicus* seeds from pods and place them in a plastic tube (*see* **Note 6**).

2. Add sulfuric acid to the tube so that seeds are completely soaked.

3. Mix well by vortexing and leave the seeds soaked for 3 minutes.

4. Eliminate the sulfuric acid by pipetting and rinse the seeds with sterile water for 10 min.

5. Repeat step 4 twice.

6. Under a biological hood, take individual seeds with flame-sterilized tweezers and place them on Agar/H_2O plates. Put approx. 5 seeds per plate.

7. Incubate plates containing the sterilized seeds in the dark at 22°C for 4 days, then put them in the light at the same temperature until the cotyledons become green (*see* **Note 7**).

3.1.4 Millipore sandwich method

The Millipore sandwich method is the technique of choice to obtain clean and intact *G. margarita* extraradical mycelium, allowing at the same time the collection of colonized root portions. The entire procedure should be performed under a biological hood.

1. Fill the Magenta boxes with quartz sand for 1/3 of their volume. Autoclave for 20 min at 120°C.

2. Pick an autoclaved cellulose nitrate membrane with flame-sterilized tweezers and place it on an Agar-H20 plate to let it moisten.

3. Take a germinated *L. japonicus* seedling (*see* subsection **3.1.3**) and place it on the membrane, with the shoot apex going beyond the edge.

4. Collect with a pipette 20-25 *G. margarita* spores and place them below the seedling rootlet; eliminate the excess liquid by pipetting.

5. Pick a second cellulose nitrate membrane with flame-sterilized tweezers and superpose it to the first one to close the sandwich.

6. Open the Magenta box and soak the sand with the 1:2 diluted modified Long Ashton solution.

7. With long, thick laboratory tweezers dig a groove in the sand, at the center of the Magenta box.

8. With long, thick laboratory tweezers take the previously prepared sandwich from the Agar-H_20 plate and place it vertically in the groove, with the plantlet apex upside. Gently close the groove and soak again with the 1:2 diluted modified Long Ashton solution (*see* **Note 8**).

9. Eliminate the excess of 1:2 diluted modified Long Ashton solution by pipetting and close the Magenta box.

10. Incubate the Magenta boxes in a climatic chamber with a photoperiod of 16 h and a temperature of 23°C during the day and 21°C during the night.

11. After 30 days, disassemble the sandwiches and collect the material under a stereomicroscope.

12. For the external mycelium, peel the root surface and place the collected hyphal bundle in tubes; for mycorrhizal roots, cut the root fragments in small pieces with a sharp scalpel and collect the material in tubes. Freeze the samples in liquid nitrogen and store them at -80°C until use.

3.1.5 *In vitro* fungal propagation under root organ culture (ROC) conditions

Root organ cultures can be used to monoxenically produce *in vitro G. margarita* BEG34 spores and mycelium (*see* **Note 9**).

1. Propagate clones of root-inducing T-DNA-transformed roots previously established by subculturing them every 4 weeks on minimal (M) medium in round Petri dishes (9 cm diameter). Keep the dishes in the dark at 26°C.

2. Using flame-sterilized tweezers, transfer a 4-5 cm long T-DNA transformed root fragment in the center of a round Petri dish (9 cm diameter) containing M medium. Gently sink the root explant below the medium surface with tweezers to avoid desiccation.

3. Transfer about 10 sterilized *G. margarita* spores in the plate, all around the transformed root explant.

4. Carefully seal the dishes with Parafilm and incubate them in the dark at 26°C.

5. A new spore generation is produced within 1.5-2 months as a result of mycorrhizal colonization. To keep the spores sterile, collect them under a biological hood using flame-sterilized tweezers.

3.2 Morphological analyses

3.2.1 Transmission electron microscopy

In order to preserve fungal structures and organelles, as well as the small endobacteria, single spores were processed by using cryo-methods, that is, high-pressure and freeze-substitution preparation. Subsequently, spore samples were infiltrated with Epon/Araldite resin and then embedded in resin blocks. For details on the cryo-preparation and the subsequent resin infiltration and polymerization refer to *(8)*. In this section, we provide details on the processing of the samples for transmission electron microscopy starting from the sectioning of the resin blocks.

1. Pre-warm the hot plate to about 50°C.

2. After embedding, the resin blocks are sectioned by using an ultramicrotome. Cut the blocks into semithin sections (1 µm) with a glass knife.

3. Place the sections on a microscope slide. Stain the sections with 1% toluidine blue (w/v). Let the microscope slide dry on the hot plate (about 50°C). Observe the stained sections under a light microscope for the orientation of the sample within the block. Select a small area of the section for ultrathin sections.

4. Cut the selected area of the block into ultrathin sections (70 nm) with a diamond knife. Treat ultrathin sections as floating sections until the end of the counterstaining step.

5. Prepare a humid chamber to prevent the sections from drying out. Place a wet paper towel inside a covered Petri dish (Ø 9 cm). Put a spot plate on the paper towel.

6. Fill a spot plate well with 500 µL of uranyle acetate solution. Lay down the sections on the solution for 20 min. Cover the Petri dish and put it on the hot plate (about 50°C).

Place a piece of aluminium foil on the cover of the dish to keep the samples in the dark during the staining.

7. Rinse twice the sections in water for 10 min.

8. Fill a spot plate well with 500 µL of lead citrate solution. Lay down the sections on the solution for 2-3 min. In order to prevent excessive lead citrate precipitation by exposure to CO_2, add NaOH pellets (~10 g) near the spot plate. Cover the Petri dish during the staining.

9. Rinse twice the sections in water for 10 min.

10. Mount the sections on a 200- or 300-mesh copper grids.

11. Observe under a transmission electron microscope (*see* **Fig. 1a, b**).

3.2.2 Confocal microscopy

Prepare a fresh aliquot of the SYTO 9® Green-Fluorescent Nucleic Acid Stain working solution (1:1000 dilution in ultrapure water). Store on ice and protect from light.

Spore processing

1. Transfer sterilized spores (1-3 spores per slide) on a microscope slide (*see* **Note 10**).

2. Add a 40-100 µL drop (depending on the size and number of spores) of SYTO 9® directly on the spores.

3. Add a large cover slide covering the entire microscope slide. Slightly press the cover slide down until you crash the spores.

4. Incubate the microscope slide for 5 min in the dark.

5. Observe under a confocal microscope (*see* **Note 11**) (*see* **Fig. 1c**).

Bacterial suspension processing

1. Transfer 10 µL of filtered bacterial suspension on a microscope slide (*see* section **2.6**).

2. Add 10 µL drop of SYTO 9® directly on the bacterial suspension and mix gently by pipetting.

3. Add a large cover slide covering the entire microscope slide.

4. Incubate the microscope slide for 5 min in the dark.

5. Observe under a confocal microscope (*see* **Note 11**).

3.3 Molecular analyses

In order to avoid contaminations carry out all steps under a biological hood (unless indicated otherwise). Prior to use, clean all the instruments (*i.e.* micropipettes, tube racks, centrifuge, etc.). Use sterile filter tips.

3.3.1 DNA extraction

Rapid DNA extraction

1. Prepare a fresh aliquot of extraction buffer with 10X PCR buffer:ultrapure water (1:1 dilution).

2. Place one to ten sterilized spores in 1.5 mL tube.

3. Crush the spore in a volume of 30 µL (single spore), 50 µL (pool of 5 spores) or 70 µL (pool of 10 spores) of freshly prepared extraction buffer.

4. Incubate crashed spores at 95°C for 15 min.

5. Centrifuge the crude extract at 16,000 g for 10 min.

6. Collect the supernatant and store -20°C.

CTAB-based DNA extraction

The procedure should be performed under a chemical hood.

1. Pre-warm water bath at 65°C.

2. Prior starting extraction, add 1% (w/v) PVP to the 2X CTAB. Prepare 1 mL per sample of extraction buffer and pre-warm to dissolve PVP at 65°C.

2. Place one to ten sterilized spores or add up to 100 µL of bacterial suspension (*see* section **3.6**) in a 2 mL tube.

3. Add 900 µL of extraction buffer, 10 µL of proteinase K (10 µL/mL) and 3 µL of RNase (25 ng/µL). Crush the spore(s) (if extracting from spores) with a 1.5 mL plastic pestle.

4. Incubate in the water bath at 65°C for 1 h.

5. Centrifuge at 9,500 g for 10 min.

6. Transfer the supernatant in a new 2 mL tube without disturbing the pellet (if present).

7. Add 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1). Mix by inverting the tube until the solution becomes lactescent and homogeneous.

8. Centrifuge at 6,000 g for 10 min.

9. Transfer the supernatant in a new 2 mL tube without disturbing the interface.

10. Add 1 volume of chloroform. Mix by inverting the tube 10-15 times.

11. Centrifuge at 6,000 g for 10 min.

12. Transfer the supernatant in a new 1.5 mL tube without disturbing the interface.

13. Add 2/3 of the volume of cold 2-propanol. Mix by inverting the tube 10-15 times.

14. Incubate the tube on ice for 30-40 min.

15. Centrifuge at 9,500 g for 10 min.

16. Carefully discard the supernatant carefully to avoid dislodging the pellet.

17. Add 200 µL of 70% ethanol.

18. Centrifuge at 9,500 g for 1 min.

19. Remove the supernatant without disturbing the pellet.

20. Resuspend the pellet in 30-50 µL of ultrapure water.

3.3.2 PCR

1. Carry out individual PCR reactions in a final volume of 20 µL containing 1X Phusion® HF Buffer, 375 µM of each dNTP, 750 nM of each primer (*see* **Tab. 1**), 0.02 U/µL Phusion® DNA Polymerase, 1-4 uL (from rapid DNA extraction) or 40-50 ng (from CTAB extraction) DNA template. Bring the mix to the final volume with ultrapure water.

2. Use the following cycling conditions:

- Initial step of 98°C for 4 min.

- Cycles (cycle conditions vary according to the primer pair used):

- CaGgADf-CaGgADr: 35 cycles at 98°C for 13 s, 69°C for 30 s, 72°C for 55 s
- GlomGIGf-GlomGIGr: 30 cycles at 98°C for 10 s, 58°C for 25 s, 72°C for 30 s
- 109F-1184R: 30 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 45 s (*see* **Note 12**)
- AML1-AML2: 35 cycles at 98°C for 10 s, 58°C for 30 s, 72°C for 35 s
- ITS1f-ITS4: 35 cycles at 98°C for 10 s, 57°C for 30 s, 72°C for 30 s
- Efgigf-Efgig2r: 30 cycles at 98°C for 10 s, 60°C for 25 s, 72°C for 20 s
- rpoBf-rpoBr: 35 cycles at 98°C for 10 s, 60°C for 30 s, 72°C for 35 s

- Final extension step of 72°C for 7 min.

3. Purify PCR products directly from an amplification reaction or extract DNA fragments from agarose gel by using Wizard® SV Gel and PCR Clean-Up System following the manufacturer's instruction.

4. Clone purified PCR products using the pGEM®-T Easy Vector System following the manufacturer's instruction (*see* **Note 13**).

5. Insert the cloned vector into One Shot® TOP10 Chemically Competent *E. coli* following the manufacturer's instruction.

6. Plate and grow transformed *E. coli* cells in the selective medium containing ampicillin.

7. Screen clones for insert length by PCR. Select positive clones.

8. Sequencing.

3.3.3 Real-time qPCR

Real-time qPCR is widely used for cultivation-independent detection and quantification of microorganisms. The estimation of the starting target quantities based on the amplification threshold cycle in each sample allows microbes (or nuclei for multinucleate organisms) to be quantified when single-copy genes are considered. The present application of qPCR can be used to quantify the abundance of endobacteria in a fungal sample, to determine the bacterial–fungal ratio (number of endobacteria vs number of fungal nuclei detected) and to relatively quantify different endobacterial populations when simultaneously present in a fungal sample (*see* **Note 14**). In order to avoid contaminations carry out all steps under a biological hood. Use sterile filter tips.

1. Prepare serial dilution of the plasmids containing the target DNA sequences (*see* subsection **3.3.4**). Quantify plasmids with the Qubit® 2.0 fluorometer and estimate the copy number/μl based upon the molecular weight of the template. Generate serial plasmid dilutions so that a 10^6 to 10^1 plasmid copies are present in 1 μL of sample solution (*see* **Note 15**).

2. Serially dilute by 10 fold the fungal sample(s) to be tested in qPCR (*see* **Note 16**).

3. Carry out individual real-time qPCR reactions in a final volume of 20 µL containing 2X real time mix, 150 nM of each primer (*see* **Tab. 1**) and 1 µL of appropriate DNA dilution. Bring the mix to the final volume with ultrapure water. Prepare three technical replicates for each sample.

4. Use the following cycling conditions: initial step of 95°C for 3 min, 40 cycles of 95°C for 15 s followed by 60°C for 40 s, with fluorescence measurement during the 60°C step (*see* **Note 17**). At the end of the amplification add a melting curve analysis as follows: 55 to 95°C with a heating rate of 0.5°C per 10 s, with continuous fluorescence measurement (*see* **Note 18**).

5. As a first qPCR run, perform a standard curve using plasmid serial dilutions to calculate the PCR efficiency. Do the same with the serially diluted fungal sample(s) to check that PCR efficiency is comparable to that obtained from plasmid standards (*see* **Note 19**). Those fungal sample(s) dilutions falling into the standard curve dynamic range can be selected for the quantification of the endobacteria/fungal nuclei (*see* **Note 20**).

6. Perform the quantification assay. Set up the qPCR plate so that both fungal sample(s) and plasmid serial dilutions are amplified in the same run. The number of target DNA sequences present in each PCR mixture is calculated by comparing the crossing points of the sample TCs with those of the standard plasmids using the StepOne™ software. If diluted fungal sample(s) were used for the quantification run, multiply the figure obtained by the dilution factor to retrieve the actual number of target DNA sequences present in the starting sample. If diluted fungal sample(s) were used for the quantification run, multiply the figure obtained by the dilution factor to obtain the actual number of target DNA sequences present in the starting sample.

3.4 Fluorescence *in situ* hybridization (FISH)

1. Prior to hybridization, fix sterilized spores in fresh and cold 4% paraformaldehyde buffered with PBS. Incubate the spores at 4°C for 3-6 h or, alternatively, at room temperature for 1-2 h.

2. Remove the fixative and wash the spores three times in 1X PBS. Process the spores immediately for the next hybridization steps, or suspend them in 50% ethanol in 1X PBS and store at -20°C until use.

3. Prepare a 2% agarose solution in ultrapure water (w/v) in a small autoclaved flask.

4. Transfer and immobilize fixed sterilized spores (*see* **Note 10**) on 8-well microscope slide with a 20-30 µL drop of 2% agarose (1-3 spores per well).

5. Dehydrate immobilized spores: plunge the entire microscope slide in an ethanol series (use Coplin jars): 3 min each, first in 50% ethanol, then 75% and 100%. Let the ethanol to evaporate but avoiding desiccation of the agarose drop.

6. Crush the spores to allow the penetration of the probes into the cytoplasm during the hybridization: crush the spores by adding a cover slide on the spore-embedded agarose drop, and by pressing slightly. Gently remove the cover slide.

7. Carry out a pre-hybridization treatment with proteinase K (1 µg/mL) for 10 min: add 50-70 µL of proteinase K on each well (agarose drop) (*see* **Note 21**).

8. Remove proteinase K and carry out the following steps: rinse with 1X PBS for 5 min; wash with 1% Tween20 in 1X PBS (freshly prepared) for 5 min; rinse twice with 1X PBS for 5 min.

9. Pre-warm the hybridization buffer to 46°C.

Carry out the next steps in the dark and avoid exposing probes to the light.

10. During the pre-hybridization steps freshly prepare the hybridization buffer (at 35% formamide stringency) in a 2 mL tube, one tube per microscope slide, as follows:

- 10700 µL of 100% formamide (final concentration 35%) (*see* **Note 3**)
- 11200 µL of 20X SSC (final concentration 2X)
- 12100 µL of Denhardt's solution (final concentration 1.25X)
- 131000 µL of ultrapure water (according to the volume of formamide)

Store on ice.

11. Deposit a 60 µL drop of the hybridization buffer on each well. Add 3 µL of each probe (*see* **Tab. 2**) at the working concentration of 50-70 ng/µL per well, directly on the surface of the drop. Avoid using more than three probes (labeled with different fluorochromes) at the same time (*see* **Note 22**). Gently mix with a pipet tip without disturbing the attached agarose drop.

12. Prepare the humid chamber to prevent probe and buffer evaporation during the hybridization: fold a paper towel and place it into a 50 mL tube. Pour the towel with the remaining hybridization buffer.

13. Place the microscope slide horizontally inside the 50 mL tube, over the moist towel, and close it tightly (the humid chamber must not dry out). Incubate in the hybridization oven at 46°C for 1 h and 30 min.

14. After hybridization, remove the hybridization buffer and rinse the samples twice with 2X SSC for 10 min and once with 0.1X SSC for 10 min. Let the microscope slide dry vertically.

15. Mount the microscope slide with a 20-30 µL drop of DABCO per well. Add a large cover slide to cover all wells (*see* **Note 23**). Remove excess DABCO.

16. Observe the microscope slide under a confocal microscope (*see* **Note 11**) (*see* **Fig. 1d**). Store the microscope slide at -20°C in the dark for several months.

3.5 Bacterial enrichment for genome sequencing

1. Distribute 1000-1200 sterilized spores in 1.5 mL tubes (about 100 spores per tube) (*see* **Note 24**).

2. Resuspend the spores in 400 µL of 0.9% NaCl.

3. Crush the spores using a 1.5 mL plastic pestle until the spore walls are well-smashed and the 0.9% NaCl becomes opaque and with a slightly pasty consistency. Use the same pestle to crush spores from the same AMF isolate.

4. Bring final volume to 1 mL, adding 600 µL of 0.9% NaCl.

5. Using flame-sterilized forceps, place the 3 µm filter in the filter holder and connect the syringe to the filter holder. Use a new 1.5 mL tube to collect the filtrate.

6. Transfer the suspension into the syringe and filter the crushed spores. Push thoroughly twice in order to allow the bacteria to pass through the filter pores. Remove the filter and place a new one in the filter holder. Repeat the passage with the other 100-spore batches. Maintain the tubes separated.

7. Centrifuge at 9,500 g for 10 min.

8. Gently remove the supernatant to avoid losing the pellet.

9. Resuspend the pellet in 30 µL of 0.9% NaCl.

10. Stain with SYTO 9® 10 µL of filtered bacterial suspension and observe under a confocal microscope (*see* subsection **3.2.2**). Fungal nuclei should not be present.

11. Treat bacterial suspension with RQ1 RNase-Free DNase A according to the manufacturer's instruction: incubation at 37°C for 30 min followed by 10 min at 65°C to inactivate the enzyme. The 100-spore batch tubes are still separated. Store at -20°C.

12. Pool the bacterial suspension and extract genomic DNA with a CTAB-based method (*see* subsection **3.3.1**).

13. Check DNA extraction for fungal contamination using primers specific for AMF and bacteria (*Ca*Gg and/or Mre) (*see* subsection **3.3.1**). Fungal primers should not provide any PCR amplification (*see* **Fig. 2**).

14. Check quantity and quality of extracted genomic DNA.

15. Sequencing.

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Notes

Note 1 *Trifolium repens* is used here as host plant for *G. margarita* propagation since it has a small size, suitable for climatic chamber cultivation, and provides a good yield in terms of AMF spores in a relatively short time. However, other host plants, such as *Sorghum bicolor* and leek (*Allium porrum*), can be used with success.

Note 2 *Cichorium intybus* T-DNA-transformed roots are used in this protocol, and they have been obtained as described by Fontaine and colleagues *(19)*. However, both *Daucus carota* and *Medicago truncatula* transformed roots can be used to set-up *in vitro* cultures of AMF, and the protocol to obtain the ROCs of such plant species is described by Bécard and Fortin *(17)* and Boisson-Dernier and colleagues *(20)*, respectively.

Note 3 Avoid to subject formamide to freeze-thaw cycles. After thawing an aliquot, it should be stored at 4°C and shortly used. Adjust the concentration of the formamide depending on the stringency necessary for the used probes.

Note 4 If, after collection, the spores still look very dirty (*i.e.* several residues are attached to the spore surface), a mild sonication can be added prior to perform the sterilization (for not more than 30-40 sec).

Note 5 Depending on the spore amount being sterilized the suitable tube should be chosen. As an example, use 1.5 mL tubes to sterilize individual batches of 100 spores each, and 50 mL tubes when groups of 1000 spores are treated together.

Note 6 Depending on the seed amount being sterilized the suitable plastic tube should be chosen. As an example, use 2 mL tubes to sterilize individual batches of 20 seeds each, and 15 mL tubes when up to 100 seeds are treated together.

Note 7 After 4 days of germination in the dark, check whether the germination occurred and, if so, place the Petri dishes in the light, otherwise wait two-three days more. The seedlings are ready to be transplanted when cotyledons become green. From that moment, they can be kept in the Petri dish prior to use for at most one week.

Note 8 The sandwich, composed by two cellulose nitrate membrane containing the *L. japonicus* seedling and the *G. margarita* spores should be handled with extreme care; it should be placed in the Magenta box so that approximately 2/3 of its height is embedded in the sand.

Note 9 The mycorrhizal efficiency is fast reducing in subsequent ROC cycles for this specific AMF isolate. Furthermore, the population of the endobacterium *Candidatus* Glomeribacter gigasporarum is dramatically reduced in successive spore generations obtained with this cultivation method, and this effect is further amplified when single spore inocula are employed *(7)*. Thus, it is recommended not to perform more than one ROC cycle to monoxenically produce *G. margarita* BEG34, unless the aim of the experiment is to obtain a cured line of the fungus, which is devoid of endobacteria *(7)*.

Note 10 When transferring the spores on a microscope slide, remove with a micropipette or absorb with blotting paper the remaining liquid (*i.e.* PBS, ethanol-PBS mixture, water).

Note 11 FITC and SYTO 9® fluorescence is excited at 488 nm and imaged with an emission window at 500 – 540 nm. Cy3 fluorescence is excited at 546 nm and imaged at 550 – 600 nm. Cy5 fluorescence is excited at 633 nm and imaged at 640 – 700 nm.

Note 12 If the primer pair 109F-1184R *(5)* primer pair does not succeed, use a semi-nested PCR approach. Carry out a first PCR with 109F and 1387R [5'-GGGCGGWGTGTACAAGGC-3', *(21)*]: cycling conditions were the same mentioned for 109F-1184R but with 55 s of extension in the cycles. Then apply a semi-nested PCR using the reverse primer 1184R: cycling conditions were the same mentioned for 109F-1184R but with 27 cycles. Use semi-nested approach for particularly difficult templates (*i.e.* templates from scarce or poor quality starting material).

Note 13 Certain DNA polymerases add a single adenine to the 3´ends of amplified DNA fragments. The pGEM®-T Easy linearized Vector contains a single 3´terminal thymidine at each end which binds to the A overhang added by the polymerase. However, the Phusion® DNA Polymerase used in this protocol, as the other DNA polymerases that have a proofreading function, produce greater than 95% blunt-end fragments. Thus, PCR fragments generated with such proofreading enzymes should be tailed at 72°C for 15 min with dATP (200 µM final concentration) prior to cloning into the pGEM®-T Easy Vector.

Note 14 The primer pairs GlomGIGf-GIGrA *(10, 7)* and RpoBRTf-RpoBRTr *(11)* were used to detect and quantify *Ca*Gg inside *G. margarita* BEG34, whereas the primer pair Efgig2f-Efgigr *(11)* was used to target the fungal host DNA. The same qPCR approach was used to relative quantify the two bacterial populations (Mre and *Ca*Gg) hosted inside *G. margarita* CM23, using the primer pairs CMsAD1f-CMsAD2r *(8)* and CaGgAD7f-CaGgAD6r *(8)* designed to specifically target the 16S rRNA gene of Mre and *Ca*Gg, respectively.

Note 15 Since the endobacteria AMF are to date considered unculturable microbes, like their fungal host, plasmids carrying the target DNA inserts were used for the construction of the standard curve for each target gene.

Note 16 The maximum dilution to be used in qPCR assays depends on the concentration of the starting material. For the quantification to be reliable, the fungal samples to be

quantified must generate threshold cycles (TCs) that fall in the dynamic range established with the standard curve generated by plasmid amplification (*see* **Note 18** and **19**).

Note 17 qPCR primers here described were designed so that their melting temperature is between 65 and 70°C and the amplified fragment for each primer pair is comprised between 80 to 150 bp. If these parameters are not respected, change annealing temperature and time accordingly.

Note 18 Melting curve analysis is necessary to assess that only a target-specific amplification occurs, and the absence of primer-dimer formation.

Note 19 The amplification efficiency (E) can be obtained from the slope of the generated standard curve using the following formula: E = (10^(-1/slope)-1)*100. This value should be between 90% and 105% for each primer pair and on each tested template (whether coming from plasmid or from total DNA extraction). Since the precision of microbial quantification using qPCR relies on the assumption that the unknown sample and the standard solutions share a comparable PCR efficiency, this should be verified prior to perform qPCR quantification.

Note 20 The qPCR output recorded for each sample is represented by threshold cycle (TC), which is the intersection between an amplification curve and the threshold line in the qPCR graph. The dynamic range represents the TC interval in which the linearity of the target quantity with the TCs has been verified for those specific reaction conditions, and within which the absence of an inhibition effect and the sensitivity of the amplification are assessed. Thus, the fact that the sample TCs fall in the dynamic range established with the standard curve assures the reliability of the quantification.

Note 21 Prior to hybridization, prepare negative controls treating the samples with RNase A (40 µg/µL).

Note 22 In addition to *Ca*Gg- and/or Mre-specific probes, use a non-specific probe, such as the universal bacterial probe EUB338 *(18)*, as positive control. Use also a negative control probe which specifically targets other bacterial taxa, such as the *Buchnera*-specific probe ApisP2a *(22)* Koga *et al*., 2003). As further negative control, use non-sense probes, such as

the probe non-BLOsADf2, which is the reverse complement of the probe BLOsADf2 *(12)*. Non-sense probes have no known rRNA target, thus ensuring that non-specific probe incorporation into the samples does not occur: they must not provide any fluorescent signal.

Note 23 DABCO drops should spread when the cover slide is placed. If not, slightly press the cover slide down until DABCO is homogeneously distributed. Due to the thickness of the agarose drops, it could happen that the cover slide does not adhere well to the microscope slide. If so, to avoid DABCO and samples from drying out, use nail polish to seal the agarose drop-formed space.

Note 24 The number of spores to be used as starting material to carry out the bacterial enrichment can vary depending on the goal of the experiment (*i.e.* bacterial genome sequencing, FISH on bacterial suspension, DNA extraction, etc.), the abundance of the endobacteria within the spores, and the size of the spores. This protocol describes the steps necessary to prepare the material for the genome/metagenome sequencing of the endobacteria from *Gigaspora margarita* (BEG34 and MR104) and *Racocetra verrucosa* (VA105B) isolates. 1000 (for BEG34) - 1200 (for MR104 and VA105B) spores used as starting material allow the obtainment of about 1 ug of enriched bacterial DNA. *G. margarita* and *R. verrucosa* produce relatively big spores (mean spore Ø 321 and 308 µm, respectively), but different AMF isolates or species could require a lower/higher spore number to be used as starting material for a bacterial enrichment and following genome/metagenome sequencing.

Table 2
List of the oligonucleotide probes used in FISH experiments

Target gene	Organism	Probe	Probe sequence (5′–3′)	Fluorochrome	References
16S rRNA gene	*Ca* Gg	CaGgADf1	CTATCCCCCT CTACAGGAYAC	Cy5	Desirò et al. [9]
	Mre	BLOsADf2	ATCCRTAGACC TTCMTCCTTC	Cy3	Desirò et al. [15]
	Bacteria	EUB338	GCTGCCTCCC GTAGGAGT	Fluorescein	Amann et al. [26]
	Buchnera	Apis2Pa	CCTCTTTGGG TAGATCC	Fluorescein	Koga et al. [27]
None	None	non-CaGgADf1	GTRTCCTGTAG AGGGGGATAG	Cy5	Desirò et al. [9]
		non-BLOsADf2	GAAGGAKGAAGGT CTAYGGAT	Cy3	Desirò et al. [15]

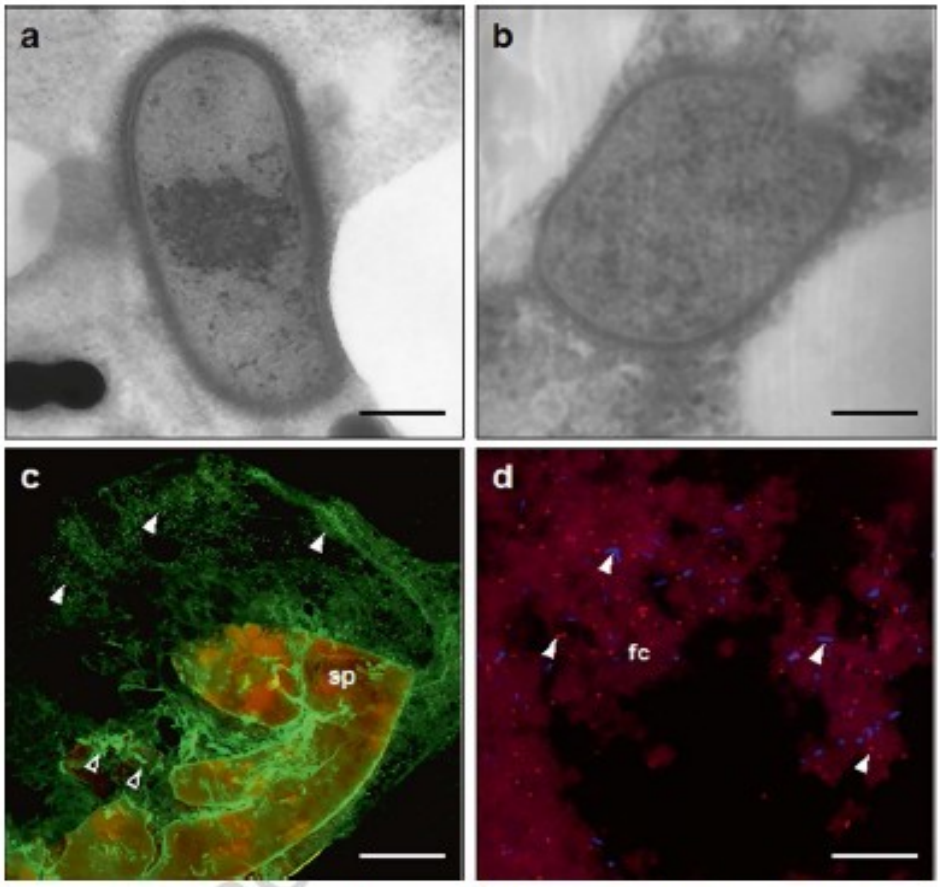

Fig. 1 Transmission electron and confocal microscopy of *Gigaspora margarita* (**a**, **c**, **d**) and *Rhizophagus clarus* (**b**) spores. Ultrastructure of (**a**) the rod-shaped *Candidatus* Glomeribacter gigasporarum and (**b**) the coccoid Mollicutes-related endobacterium as seen under a transmission electron microscope. (**c**) Crushed *G. margarita* spore (sp) after staining with SYTO 9®: the cytoplasm spreads over the slide forming a halo rich in endobacteria (*arrowheads*). Fungal nuclei (*empty arrowhead*) are trapped inside the cytoplasm. (**d**) FISH on a crushed spore of *G. margarita*: the double labeling with the *CaGg*-specific probe CaGgAD1f (*blue*) and the Mre-specific probe BLOsADf2 (*red*) confirms the simultaneous presence of the two endobacterial types in the same AMF spore; bacteria are seen as rod-shaped or coccoid fluorescent spots (*arrowheads*). Fungal cytoplasm (fc). Scale bars, (**a**) 0.13 μm; (**b**) 0.12 μm; (**c**) 150 μm; (**d**) 13 μm

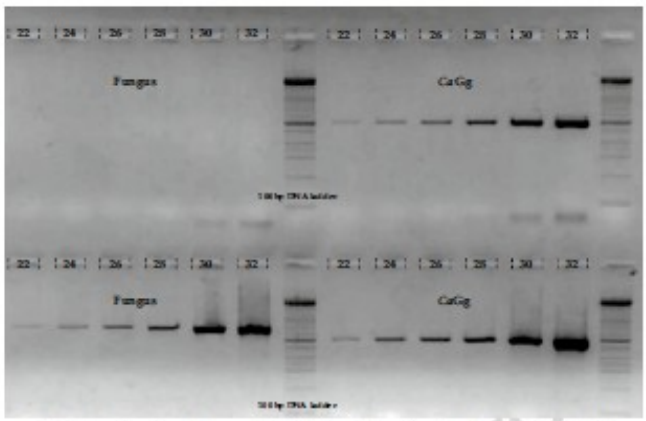

Fig. 2 Agarose gel electrophoresis patterns of the PCR amplification targeting the 18S rRNA gene of *G. margarita* (*left*) and the 16S rRNA gene of *Ca*Gg (*right*). The fungal and bacterial detection was carried out using the primer pairs AML1-AML2 [23] and GlomGIGf-GlomGIGr [13], respectively (*see* Subheading 3.3.2). After a rapid DNA extraction (*see* Subheading 3.3.1), the samples in the upper part of the gel were subjected to bacterial enrichment prior to amplification (*see* Subheading 3.5), whereas the ones in the lower part of the gel were directly amplified. Six subsamples for each assay were prepared. Each subsample was amplified with a different number of cycles (22, 24, 26, 28, 30, 32, respectively). As expected, the greater was the cycle number, the higher was the amount of amplicons obtained. Interestingly, any fungal amplification was observed from the samples enriched in endobacteria, suggesting a limited or absent carryover of fungal nuclei. These results confirmed the efficiency of the bacterial enrichment procedure here described